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Principles of Bone Marrow Transplantation (BMT): Providing Optimal Veterinary and Husbandry Care to Irradiated Mice in BMT Studies

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Bone marrow transplantation (BMT) is the treatment of choice for many leukemias, solid tumors, and metabolic diseases. The field of bone marrow research is highly dependent on in vivo experimentation, because in vitro techniques do not mimic these complicated in vivo systems. Therefore, understanding the medical and husbandry care needs of these transiently immunodeficient bone marrow recipient animals is crucial for researchers, veterinary and animal care personnel. Here we discuss the principles of bone marrow transplantation, mouse pathogens that can interfere with transplantation research, and important husbandry and veterinary practices for mice that may help to minimize unnecessary infections during the transplantation process. Whole-body irradiation is one of the most common tools for myeloablation of the recipient's bone marrow. We discuss the crucial role of the irradiator for BMT research and the importance of aseptic husbandry practices to lessen the possibility of the irradiator for being a source for disease transmission. Finally, we discuss some important guidelines for Institutional Animal Use and Care Committees reviewing irradiation and BMT protocols.

Abbreviations: BMT, bone marrow transplantation; GVHD, graft-versus-host disease; HVG, host versus graft; IACUC, Institutional Animal Care and Use Committee; SPF, specific pathogen-free; TBI, total body irradiation.

The use of animals for research comprises approximately 50% of the NIH research-funded activities.[19] The field of bone marrow transplantation (BMT) historically has been highly dependent on in vivo models. In terms of numbers, the mouse is the mammal used most frequently for BMT studies. Murine models have clear advantages in that they share similarities in physiologic and pathologic traits with other mammals, including humans. The small mass of mice, their large litter sizes, short pregnancy period, and availability of diverse stocks and strains as well as transgenic, knockout, and knock-in lines have made them one of the most valuable and versatile experimental animal models for both human and veterinary biomedical research. During BMT, recipient mice may receive a genetically identical bone marrow graft, or, often, a genetically disparate graft. If genetically disparate BM grafts are transplanted, a severe immune reaction stemming from the donor cells attack the hosts' tissues. However, if the host immune system is not pretreated (that is, immunosuppressed to some degree), failure of engraftment or graft rejection (of the donor BM) may occur. Many methods are used to ablate the immune system. The easiest and most commonly used method experimentally is total-body irradiation (TBI), which is achieved by placing the mice in specifically designed irradiators; the dose of whole-body gamma irradiation causes the animals to become either transiently or chronically immunosuppressed. Because of the animal's weakened immune system, strict veterinary and husbandry care requirements are needed to ensure the well-being of these animals.

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In this review, we discuss the basic principles of BMT, transplant-related complications, the role of animal irradiators, specific husbandry and veterinary care needs of animals undergoing BMT, the potential lethal effects of infectious agents that otherwise would be considered inconsequential, and some common animal care and use concerns that must be addressed when working with irradiated and BMT animals.

Principles of Bone Marrow Transplantation

More precisely, the process of BMT should be termed hematopoeitic cell transplantation or hematopoietic stem cell transplantation, because the stem cells responsible for reconstituting the immune system can now be harvested directly from the circulation. Currently, most transplants deliver peripheral-blood–mobilized stem cells and not cells harvested directly from the BM by aspiration. Another source of stem cells used currently is the umbilical cord.[103] During BMT, a donor inoculum is given to a recipient. The inoculum contains pluripotent hematopoietic stem cells, as well as more mature hematopoietic cells arising from the myeloid, lymphoid, and erythroid lineages. These hematopoietic cells are harvested from bone marrow (for example, the iliac crest or long bones) or from the circulation after administration of granulocyte colony-stimulating factor or other growth factors that mobilize these cells to the peripheral circulation. In small animal models such as mice, the bone marrow from a donor mouse is the most common source of stem cells; however, in larger species such as dogs, pigs, and primates, peripheral blood stem cells can be harvested more easily due to the greater blood volume of the animals. Two types of progenitors reconstitute the recipient's immune system after hematopoietic stem cell transplantation:

short-term and long-term hematopoietic cell progenitors. Most precursors that repopulate the lymphoid and myeloid lineages soon after transplantation are short-lived.[49,73] The length of time that short-term multilineage precursors function in the recipient appears to be proportional to the lifespan of the donor species. For example, short-term precursors disappear 3 to 4 mo after transplantation in mice[49,73] but persist for 1 to 4 y in cats.[1] The long-term repopulation precursors are responsible for long-lived hematopoietic reconstitution[72-73] and therefore are considered the true pluripotent hematopoeitic stem cells (Figure 1).

By approximately day 7 after BMT, donor-derived cells such as monocytes, dendritic cells, and neutrophils can already be found in the spleen of recipient mice,[3] and by day 21 after BMT, peripheral lymphohematopoietic reconstitution of all cell lineages may be normal.[74] However many of these innate cellular effectors are yet not fully functional,[74] and therefore BMT recipient animals are still at risk of opportunistic infection at this time.

Transplantation of a genetically identical graft (syngeneic graft or autologous bone marrow) causes no rejection. With a BMT in which the donor and the recipient are genetically different (allogeneic graft), the recipient develops 'runt disease,' a syndrome that features profuse diarrhea and skin lesions. These clinical signs, caused by a response of the donor cells to the recipient's tissues, is known as graft-versus-host disease (GVHD).[26,33] The liver, skin, intestinal tract, and lymphohematopoietic system are the major targets of GVHD.[32] Not all incompatibility differences produce the same degree of GVHD.[18] The strongest posttransplantation immune reactions occur when all major histocompatibility complexes are mismatched, some of which are more immunogenic than others.[32] Many factors modulate the GVHD immune response. Preparatory regimens such as TBI and chemotherapy have been shown to cause a severe inflammatory response prior to the transplant that fuels the subsequent allogeneic responses caused by the donor graft.[36] Tissues where there is continual antigen presentation (for example, lymph nodes and gut- and mucosa-associated lymphoid tissue) have the important role of priming T cells, which consequently will migrate to the peripheral organs and tissues to cause damage. Just as with other immune responses, the downregulation of certain inflammatory cytokines such as TNFα[15] is beneficial in reducing the severity of GVHD. Although BMT results in more cures and remissions than do many other alternative treatments, approximately 40% of the patients that receive an allogeneic bone marrow transplant die secondary to transplant-related complications (such as GVHD).[18] In response, an intense clinical research effort is being undertaken to study safer preparatory regimens and peri- and postBMT therapies. In the translational research effort to develop safe transplant techniques and therapies that minimize rejection and GVHD, the mouse has become one of the most used experimental animals. Veterinary and animal care staff must have an understanding of the husbandry needs that these mouse models require, and the health risks that they endure, during the transplant process.

Noninfectious Transplant-Related Complications

Routes of delivery of the hematopoietic stem cell graft. In general, a BM graft is delivered through the tail vein in mice. To inject the tail veins of animals 10 to 12 wk of age and weighing about 18 to 20 g, 25-gauge needles can be used. Smaller gauge needles, though they can be used, may increase shearing of the cells in the inocula. Other sites for delivery include the retro-orbital venous sinus,[52,72] the bone marrow cavity itself,[46,54,110] and the spleen.[14] Injection into the retroorbital sinus is easier to perform, but more invasive, than using the tail vein, and it requires the recipient mouse to be anesthetized. The splenic route is not commonly used for delivering hematopoietic stem cells to repopulate the bone marrow, because it may be less successful than the other routes. The homing of stem cells to the marrow is dependent on molecules such as stromal-derived factor 1 and stem cell factor that guide the stem cells from the peripheral blood to the marrow cavity.[56] Therefore, delivery of the stem cells into the circulation (or better, orthotopically into the marrow) increases the likelihood that the cells will establish residence in the bone marrow of the new host. Bone marrow and splenocyte isolation protocols are described in Figures 2 and 3.

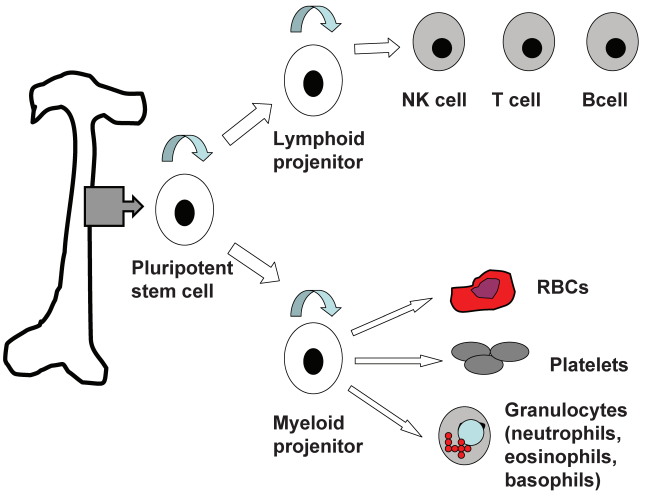

Figure 1. Schematic representation of the hematopoietic cell lineages deriving from the bone marrow.

Failure of hematopoietic cell reconstitution. Because recipients typically have had a myeloablative procedure (for example, irradiation or chemotherapy) prior to the BM transplant, these animals are at serious risk of death if the graft fails to establish itself. If the donor hematopoietic stem cells fail to engraft, recipients eventually will succumb to infection secondary to BM aplasia, anemia, or thrombocytopenia. Some reasons for unsuccessful engraftment include technical error, insufficient donor graft in the inoculum, an acquired or inherited deficiency within the donor cells, failure of donor cells to survive the procedure, and use of T-cell depleted marrow in an allogeneic transplant.

Technical error. Operator technique is crucial for successful delivery of the graft. When faced with increased recipient mortality, one must ensure that the laboratory personnel have had proper training and experience in delivering these grafts. If necessary, personnel should practice their skills by delivering saline 'inocula' into naïve animals; this opportunity could be used to demonstrate their proficiency to a veterinarian in the event of extensive grafting failures. Furthermore, the institution may want to consider developing a for-fee injection service, teaching the laboratories tail-vein injection techniques and recommending other techniques such as retroorbital injections under anesthesia (less challenging technically but more invasive than tail-vein injections).

Insufficient donor graft in the inoculum. Theoretically, a single pluripotent hematopoietic stem cell (Figure 1) is sufficient for long-term engraftment[76] and repopulation of the BM (Figure 4). However, additional short-lived 'helper' BM cells are required

Bone Marrow Isolation Protocol

Adapted from the laboratory of James L.M. Ferrara. Experimental Bone Marrow Transplantation Laboratories at the University of Michigan Comprehensive Cancer Center

1.0 Description

Isolate marrow content from femur and tibia bone cavities and T cell populations from spleen.

2.0 Materials and Reagents

Culture media: 5% to 10% fetal bovine serum in RPMI

50-mL conical tubes

60- and 100-mm culture dishes

Dissection tools (scissors, scalpel, forceps) and tray

3.0 Procedure

3.1 Euthanize bone marrow donor mouse

3.2 Pull both halves of the skin away from the incision, exposing the muscular tissue below.

3.3 Periodically spray mouse with 70% ethanol to maintain aseptic conditions during procedure.

3.4 Lift the skin on the left side of the mouse and make a small incision beneath the rib cage.

3.5 Remove both legs with scissors. Try to remove as much hair, skin and muscle as possible without compromising the marrow cavity. Remove a portion of the pelvic bone if necessary.

3.6 Place approximately 7 ml culture media in a small culture dish and approximately 35 ml media in a large culture dish in the hood.

3.7 Thoroughly scrap the tibia and femur of all fascia, connective tissue, and muscle. Separate bones at the knee joint. Store bones in media in the small culture dish.

3.8 Carefully clip the epiphysis and distal ends of each bone. Using approximately 2 ml of media taken from the large culture dish, flush bone marrow with a 27-gauge needle into the dish.

3.9 Agitate clumps of cells by using a pipette and then pass them through a 40- to 70-µm cell strainer. Be sure to rinse culture dish with approximately 10 to 15 ml fresh media to ensure all cells have been collected.

Figure 2. Bone marrow isolation protocol.

to ensure the survival of the experimental mouse during the early postBMT period. This need arises because hematopoietic stem cells require time to engraft and differentiate into the various hematopoietic lineages and because HSCs represent only about 1 in 10^5 to 10^6 bone marrow cells in the adult mouse.[30] Because of this infrequency, many groups studying HSCs supplement their mice with additional mature bone marrow cells. In general, at least 2.0×10^5 additional mature BM cells are supplemented[30,52] to ensure the survival of the recipient mouse shortly after myeloablative TBI, some references recommend larger populations (for example, 4.0 to 20.0×10^5).[72] For studies focusing on GVHD or graft-versus-leukemia effects (which is the intended goal of the donor graft being used to kill the leukemic cells), a minimum of 2.0 to 5.0×10^6 BM cells (containing both mature hematopoietic progenitor cells and pluripotent stem cells) generally are injected[16,35,44,63,82] to both syngeneic and allogeneic recipients.

Acquired or inherited deficiency within the donor cells. If donor BM stem cells have an acquired or inherited deficiency,[56,92] they will often not engraft. If the laboratory is infusing a sufficient number of cells to ensure engraftment in the recipient, they should investigate whether the donor mouse strain or line has any known deficiencies in stem cell homing molecules. If such a deficiency is present, the laboratory should consider delivering the graft orthotopically (directly into the bone marrow), further increasing the number of hematopoietic stem cells delivered, or supplementing the graft with wild-type BM cells (if the experimental design allows it).

Failure of donor cells to survive the procedure. Necrotic or apoptotic donor cells will not survive the transplant. To determine the degree of necrosis and apoptosis within the graft, the donor inoculum should be assessed with annexin V and propidium iodide.[10,71,77] Another reason for failure of donor cells to survive the procedure is development of host-versus-graft (HVG) disease, which is classic 'transplant rejection.' This failure would occur when the recipient is not myeloablated completely, either accidentally (irradiator malfunction) or purposefully (experimental design). In HVG disease, the host's surviving immune cells attack the donor cells, making it more likely that these cells will not engraft.

Use of a T-cell depleted marrow in an allogeneic transplant. Failure of engraftment may occur when the donor marrow is

Splenocyte Isolation Protocol

Adapted from the laboratory of James L.M. Ferrara. Experimental Bone Marrow Transplantation Laboratories at the University of Michigan Comprehensive Cancer Center.

1.0 Description

Separation of spleen cell subpopulations can be achieved with subsequent purification steps.

2.0 Materials and Reagents

Complete cell media: 5% FBS in DMEM containing penicillin, streptomycin, glutamine, nonessential amino acids, sodium pyruvate, and 2-mercaptoethanol

Sterile frosted microscope slides

15-ml centrifuge tube

15-mm culture dish

Dissection tools (scissors, forceps) and tray

3.0 Procedure

3.1 Pull both halves of the skin away from the incision, exposing the muscular tissue below.

3.2 In hood, carefully clip the peritoneum open above the bean-shaped, dark-colored spleen.

3.3 Carefully remove the spleen through this opening, and clip off connective tissue to remove.

3.4 Place 5 ml of media in a small culture dish. Coat rough side of two microscope slides with media.

3.5 Place excised spleen between microscope plates and gently press to pulverize tissue.

3.6 Wash splenocytes into culture dish with media.

3.7 Wash microscope slides free of residual splenocytes with additional volume (2 ml) of media.

3.8 Pipet up and down gently in culture dish to break up clumped splenocytes.

3.9 Pipet cells over a 70-μm cell strainer. This step will remove any residual fibrous connective tissue that remains attached to the spleen.

3.10 Spin cells at 1200 rpm for 5 min.

3.11 Remove supernatant, and resuspend cells in 1.0 ml of fresh warm media.

Figure 3. Splenocyte isolation protocol.

T-cell depleted.[22] Therefore, if high numbers of mouse deaths occur after BMT and failure of engraftment is suspected, whether the mice are receiving T-cell–depleted bone marrow should be investigated. If so, increasing the dose of bone marrow cells may improve survival.[4,22]

Several simple tests are available to assess specifically for graft failure. The first is to assess for lymphohematopoietic reconstitution in the peripheral blood of animals identified to be sick or moribund. A complete blood count using either automated or manual hematocytometers or staining for specific blood cell lineages by using fluorescence-activated cell sorting should be performed. Some common cell markers of interest are Gr-1/Ly6G and Ly6C for granulocytes; F4/80 and CD11b for monocytes; CD3, CD90, CD8, and CD4 for T cells; B220 for B cells, Ter119 for erythrocytes, and CD62P for platelets.[7] Most research facilities have access to automated cell-sorting machines. If failure of engraftment has occurred, the blood cell lineages will be low or absent when compared with healthy control animals. For a definitive diagnosis (recommended), assessment of bone marrow lymphohematopoietic precursors can be performed. Unfortunately bone marrow aspirates in mice are difficult to perform due to the inherent size of the host. Therefore ill mice need to be euthanized and tibias and femurs collected, decalcified, and submitted for pathologic assessment of BM precursor cells. If cell lineages are present, whether the cells are of donor or recipient origin should be investigated. If most cells are of host origin, incomplete recipient BM ablation may have occurred, thus preventing the donor BM cells from engrafting. Insufficient ablation can occur if the dose of irradiation was only partially or never given (nonmyeloablative

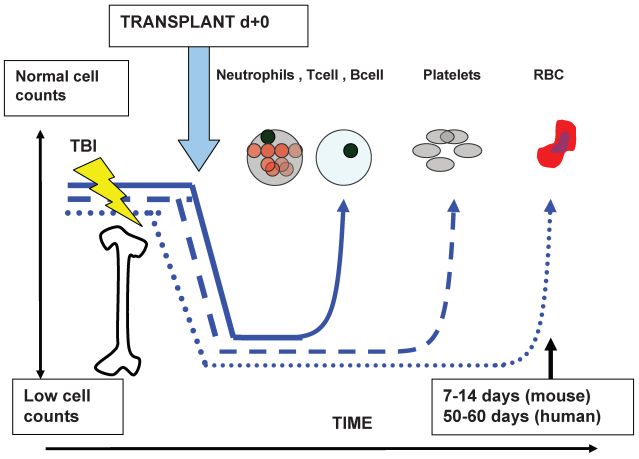

Figure 4. Schematic representation of hematopoietic reconstitution over time. After myeloablative TBI, the neutrophil and lymphocyte lineages are the first to recover after BMT. The platelet and red blood cell lineages reconstitute the peripheral circulation at a later time point. Although the order of hematopoietic reconstitution is accurate, the time for recovery may vary greatly depending on the degree of myeloablation. This time frame is influenced by the strains and ages of the mice (donors and recipients), as well as technical differences such as dose fractionation and supplemental local irradiation (for example, to the thymus). We recommend that the veterinary team discusses the expected recovery of the hematopoietic system with the laboratory performing the experiments.

regimen). Cell surface markers can be fluorescently labeled and used to track whether the resident BM cells in syngeneic transplant models are of donor or recipient origin, even though no HVG (or GVH) disease occurs in this setting. For example, in syngeneic BMT, C57BL/6 mice (Ly5.1) are often injected with congenic C57BL/6 (Ly5.2) stem cells (or vice-versa). In this context, the donor or recipient cells can be easily identified by their Ly5 (CD45 marker).[72,99] In full-mismatch allogeneic transplants, differences in the donor and recipient haplotypes ($H2^b$, $H2^d$, $H2^k$, etc.) can readily be assessed. Most importantly, in addition to identification of the origin (host or recipient) of the cells, whether the overall numbers of cells present are sufficient to ensure survival must be determined. Therefore, donor and recipient chimerism as well as the presence or absence of mature and immature lymphohematopoietic cells should be assessed from both the peripheral circulation and the BM if animal deaths are suspected to have been due to engraftment failure. If the results are unclear, the veterinary staff may suggest that the laboratory perform a no-transplant control after irradiation. In addition, in general the maximal time that transpires between irradiation, whether fractionated or given as a single dose, and delivery of the inoculum is about 3 to 10 (overnight) h after the last radiation dose.

In summary, when faced with increased recipient mortality and suspected failure of engraftment one must: evaluate the degree of experience of laboratory personnel delivering the graft; reassess the number of cells in the inoculum; investigate whether the donor mouse strain has any known molecular deficiencies that could affect homing of hematopoietic stem cells to the recipient marrow; request analyses for the viability of the grafted cells and the possibility of HVG disease; confirm the presence of sufficient donor T cells especially if the graft is allogeneic; and lastly determine the origin (donor or recipient) of the cells. As with any workup, full understanding of the experimental goals and expected outcomes must be considered prior to recommending any changes.

Irradiators and the Effects of Irradiation in Mice

Irradiators. This review will focus specifically on the effects of irradiation, which is the most commonly used method of myeloablation in the mouse. A discussion of chemotherapeutic agents (e.g., busulfan, cyclophosphamide, other alkylating agents) is beyond the scope of this review. Successful survival of a bone marrow graft requires suppression of the host's immune system in some manner to prevent HVG rejections. In addition to suppressing the host's immune system, irradiation also helps deplete the bone marrow niche of host progenitor cells, thereby allowing space for engraftment of donor stem cells. For small animals, this preparation commonly is accomplished through whole-body gamma irradiation. Irradiators vary in size depending on their intended use. Small irradiators (for example, the Mark-I irradiator from JL Shepherd and Associates, San Fernando, CA) are the size of a refrigerator and commonly are used to irradiate both cells and mice. This irradiator is limited, however, by its small chamber size, which holds only a few mice at a time for irradiation. In contrast, one commonly used larger (6600 lb) gamma irradiator (the Gammacell-40, MDS Nordion, Ottawa, ON) can be used to irradiate several dozen mice at once (Figure 5 B). Although more than 25 mice can fit inside this larger irradiatior, at the authors' institution the capacity is limited to 20 animals to avoid unnecessary overcrowding (Figure 5 A). Animals are generally irradiated for short periods of time (less than 15 min). The amount of time spent inside the irradiator varies depending on the radioisotope decay charts, amount of irradiation needed, and source of ionizing energy (that is, X-rays versus gamma rays, for which a cesium or cobalt source is needed). Irradiators (for example, Clinac 4/80 linear accelerator, Varian Medical Systems, Palo Alto, CA) also are available for even larger animals (dogs, monkeys, pigs). An important difference between the mouse-sized and large animal irradiators is that mice need not be anesthetized for irradiation. In either case, the overall scientific goal is to render the recipient partially or completely immunosuppressed with minimal animal distress.

Effects of irradiation. Briefly, ionizing radiation causes breaks in the DNA double-strand;[29,80] thus it mostly affects mitotically active cells. The DNA breaks occur in multiple sites, and damage is so severe that the cellular repair systems are unable to fix the DNA. Consequently, this damage leads to cell death through either necrosis or apoptosis. The cells in the hematopoietic system and gastrointestinal tract are extremely sensitive to irradiation because they are always mitotically active.

Like humans, all mice do not respond identically when exposed to irradiation; many biologic factors potentially can affect the murine response to ionizing radiation. For example, older humans treated for their malignancies with a myeloablative TBI regimen are more prone to develop GVH disease, compared with younger patients given a similar preparatory regimen.[98] This age-dependent effect also occurs in mice.[75] The dose of irradiation (Figure 6) and the strain of the mouse[25,42,47,48] are 2 additional factors that can dramatically affect the degree of irradiation sickness. BALB/c mice are very sensitive to irradiation.[42,50] Whereas the commonly used B6 mice can typically tolerate a radiation doses of 1000 to 1100 cGy,[25] the LD50 of TBI in BALB/c mice is about 880 cGy.[42] If given higher doses, BALB/c mice develop considerable radiation-induced sickness (lethargy, inappetance, diarrhea) that may lead to death. Therefore milder irradiation doses[87] should be used with BALB/c mice to avoid unnecessary irradiation-induced deaths. However, in recent disease studies of GVH and GVL using

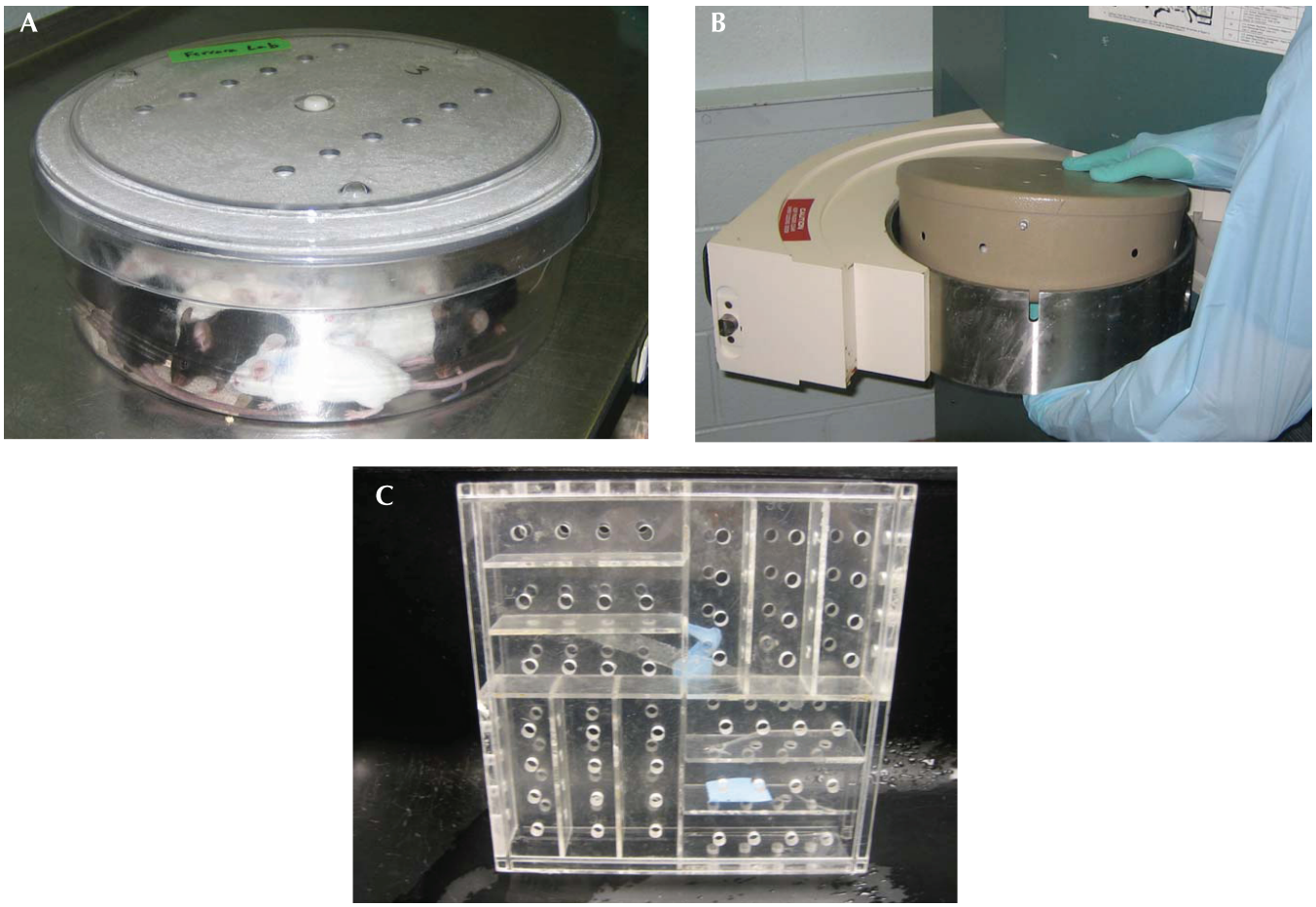

Figure 5. (A) Container with mice to be irradiated. (B) Container placed in the irradiation drum in an irradiator. (C) Acrylic irradiation holding device.

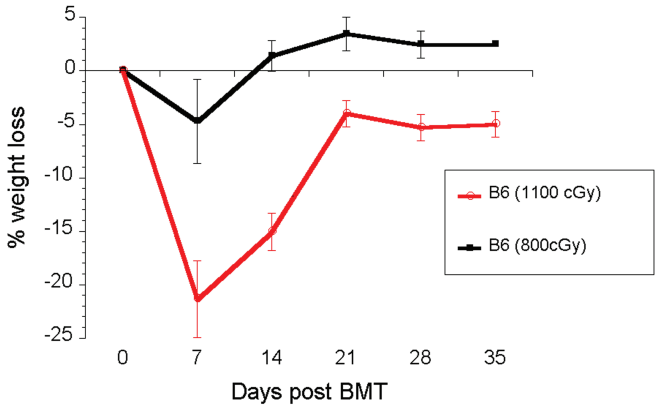

Figure 6. Syngeneic bone marrow transplant (C57BL/6 (Ly5.1)→C57BL/6 (Ly5.2)). C57BL/6 Ly5.2 mice, were given either 800 cGy total-body irradiation (in black) or 1100 cGy (in red). All mice received a tail vein inoculum comprising 1.0×10^7 bone marrow cells and 2.0×10^6 CD3 magnetic bead cell-sorted T cells from the spleen. Cells were mixed prior to injection and delivered 1 to 3 h after irradiation. All mice (n = 8/group) survived. Note the difference in weight loss based on irradiation dose.

BALB/c mice as recipients, radiation doses of 800cGy were well-tolerated.[27] Others have used similar radiation regimens in BALB/c mice.[28]

Ample documentation in the literature shows that irradiation doses of 700 to 1300 cGy are myeloablative. The higher the dose, the greater the likelihood for the animals to die secondary to irradiation-induced toxicity.[24,42,50,95] The lower the dose, the longer it may take to achieve full donor chimerism (in which the new immune system is 100% donor-derived) and the greater the chance for HVG response (in which the donor graft is rejected by the recipient's immune system). Under some conditions, doses less than 550 cGy in allogeneic BMT has prevented donor engraftment.[24] However, not all radiation regimens aim for complete myeloablation. Some studies target a stable (tolerant) mixed chimeric state, when the immune system is composed of cells from both the donor and the recipient. This goal can be achieved by using low irradiation doses (300 to 700cGy) in addition to costimulatory blockade (blocking crucial stimulatory pathways known to activate alloreactive T cells) and the use of immunosuppressive drugs.[85] Therefore, the amount of irradiation may vary widely from experiment to experiment, depending on the investigator's specific aims. In any case, the overall goal when using TBI in transplantation studies is to fully or partially immunoablate the recipient while minimizing the toxic radiation side effects. This balance is achieved by catering the amount of irradiation to the individual experimental group, and by paying close attention to the strain and age of the recipient mouse.

Irradiation causes animal morbidity through tissue damage, which in turn elicits an inflammatory response. This inflammatory response is mediated, in part, by TNFα, which is responsible for many systemic effects, including fever,

hypotension, adult respiratory distress, shock, and vascular leakage syndrome. TNFα is secreted by the damaged cells after irradiation.[31] When radiation damage to the intestinal epithelium occurs, normal intestinal bacteria and their toxins are translocated into the bloodstream.[15] These bacterial products, especially lipopolysaccharide, further enhance the inflammatory response, subsequently weakening the recipient.[17] This inflammatory milieu primes antigen-presenting cells like macrophages and dendritic cells to further secrete more TNFα. The result of this severe inflammatory cascade is the physical sickness noted in both animals and humans after irradiation. When these events are coupled with the inherent transient period of immunodeficiency, conditions are ideal for the irradiated animals (or humans) to be at risk of death secondary to infection. During this early inflammatory period after irradiation, the veterinary and husbandry staff members should closely monitor the animals and when the recipient's will be expected to lose/gain back weight (Figure 6). If death is observed in a high percentage of transplanted animals during this early period after irradiation, a clinical workup to determine the cause should be initiated. In addition to assessment of irradiation toxicity, inadequate irradiation dosages (and thus failure of engraftment) must also be considered. One method to reduce the development of illness after irradiation is to decrease the individual dose but increase the overall exposure time. Fractionating the total dose into 2 equal-half doses given at least 3 h apart has proven to cause less radiation-induced tissue damage.[104] In larger species, such as in humans, fractionation of the irradiation doses has been common practice. These radiation fractionation protocols have been developed from studies in dogs.[21,96,97]

With any therapy that has the potential to damage cellular DNA and cause immunosuppression comes the inherent risk for developing secondary neoplasias, as noted for bone marrow transplantation.[20] The most commonly documented solid neoplasm after BMT neoplasm in humans is malignant melanoma. The incidence of this type of tumor is higher in younger BMT patients. Increased doses of TBI (and thus immunosuppression) were associated with an elevated risk of hematopoietic and solid cancers.[20,61] Documentation of these effects in human subjects has been possible due to their prolonged survival after BMT and follow-up.[20,60,61,94] In 1965, scientists documented this increased incidence of neoplasia in bone marrow transplanted mice,[88] which supported the clinical findings from human transplant recipients that had undergone a similar treatment. Furthermore, similar reports of neoplasia after immune suppression appear in the veterinary literature.[13,45,59,86,107] In summary, the possibility for development of solid or hematologic tumors in long-term irradiated or chronically immunosuppressed mice must be remembered. Although most mouse BMT experiments are of short duration, the veterinary and husbandry staffs should be made aware when investigators plan to keep long-term BMT chimeric mice, because these animals are at an increased risk for tumor development and other postirradiation-specific conditions. One such additional, nonneoplastic illness seen in mice is incisor damage after nonmyeloablative TBI.[57]

Veterinary and Husbandry Care of Irradiated or Transplanted Animals

Several husbandry concepts should be considered when caring for irradiated mice before and after BMT. As mentioned previously, irradiated mice remain immune compromised for a period of time after BMT, and therefore they should be housed under strict barrier conditions. These animals should be handled only under a HEPA-filtered flow hood or in a HEPA-filtered laminar flow room. These filters can remove particles up to 0.3 microns in size, including aerosolized bacteria and fungal spores. The room that houses mice after BMT should be maintained under positive air pressure relative to the corridor, in order to minimize the risk of aerosolized pathogens entering the room. Anyone handling these animals (caretakers or research staff) should consider wearing a gown, gloves, hair bonnet, and a surgical mask. The basic goal of these efforts is to prevent the transmission of any potential pathogen from humans or the environment to the transiently immunodeficient mice.

Transplanted animals undergo a 5- to 10-d irradiation sickness period from which they generally recover within 14 d[5,16,63,64,74,83,100] (Figure 6). Early after irradiation and transplantation, recipient mice can become dehydrated, sometimes due to the diarrhea that develops from the radiation-induced damage to the intestinal epithelium, coupled with the generalized malaise due to the irradiation-induced inflammatory response. Irradiated mice must have easy access to water. A practical way to provide water is to use bottles with long (approximately 10 cm) sipper tubes, which enable the animals to get to the water source with minimal effort. Another way to provide fluids is by using a gelatinized water product [for example, Napa Nectar (SE Lab Group, Napa, CA)] in the bottom of the cages, but such products run the risk of becoming contaminated with fecal bacteria unless replaced frequently. Other alternatives include individual fluid treatment of each mouse by subcutaneous, intraperitoneal, or oral administration; however, these methods are time- and labor-consuming when dealing with large populations of irradiated mice.

The need for sterilized food and water for these mice is an issue of debate. The food should be kept dry to minimize the growth of potential fungal pathogens. The water should be ultrafiltered and purified (Milli-Q or RiOs systems, Millipore, Billerica, MA) or autoclaved. We have encountered sporadic 'pink' water (Milli-Q filtered) in bottles that, at various times, has been cultured and found to be contaminated with *Comamonas testosteroni*, *Pseudomonas alcaligenes*, *Bacillus* spp., *Enterococcus* spp., or *Ralstonia pickettii*. We did not observe an increased mortality in the animals that received the contaminated water. However, in view of these culture results, and to avoid any potential bacterial infections, we since have used filtered water acidified with hydrochloric acid and have noted a decrease in water bottle contamination. *Pseudomonas aeruginosa* was eradicated from the oral cavity of infected mice by acidifying the water.[14] The University of Missouri's Research Animal Diagnostic Laboratory suggested that maintaining the pH of the water between 2.2 to 2.5 will prevent most bacterial contaminations.[102] However, in our experience, acidifying the water to a pH below 2.7 to 2.8 can reduce drinking, resulting in dehydration. Therefore, at our institution, we target a pH of 2.9 to 3.0 so that the transplanted animals will drink the water readily. However, several bacterial species (in particular, some *E. coli* strains and *Shigella* spp.) have been documented to grow even under acidic conditions,[43,93] so bacterial contamination is still possible at pH 3. Basically, the drinking water should be as free of microbial pathogens as reasonably possible. In addition, water bottles should be changed at least once each week to minimize the possibility of contamination, regardless of the type of filtering or acidification used.

Administration of antibiotics in the drinking water may minimize bacterial contamination within the water source and potentially decrease the burden of gastrointestinal bacteria. As mentioned earlier, bacterial translocation from the intestinal tract after irradiation is a common source of systemic infection

in both human and animal BMT patients. Therefore, decontamination of the gastrointestinal tract by using oral antibiotics has been used prophylactically in humans[41] and animals. Antibiotics can be administered to the animals by individual gavage or provision of treated water; commonly used antibiotics include metronidazole, neomycin, ciprofloxacin and tetracyclines.[11,105] However, this practice has several associated risks: development of bacterial resistance to the antibiotic used, or promotion of a growth advantage to less favorable bacteria which are unaffected by the antibiotic;[2,51,55,112] inability to accurately measure the amount of antibiotic that each mouse drinks, as the daily water consumption of each mouse cannot be ensured (especially during irradiation-induced illness); and inactivation of some antibiotics shortly after dissolution or when exposed to light.[106] Therefore, the appropriate antibiotic must be selected carefully and the health of animals monitored for both antibiotic-associated illness and ineffectiveness of treatment.

Some investigators feed autoclaved rodent chow to BMT mice. No definitive human transplant studies indicate a survival advantage to feeding sterilized food to patients during their peritransplant period. However, raw fruits, vegetables, and undercooked meats traditionally are avoided (University of Michigan Hospital Bone Marrow Transplantation Unit - personal communication).[81] Although the use of sterilized food is advantageous when caring for traditionally immunocompromised mice (for example, nudes and SCIDs), to our knowledge no reports have specifically documented a positive survival advantage in BMT mice when autoclaving rodent chow. Autoclaved and nonautoclaved diets are both provided at our institution, and no differences in survival have been noted after BMT. Extracts from plants such as *Podophyllum hexandrum* (a perennial herb) and *Hypophae rhamnoides* (seaberry) are radioprotective and improve survival after lethal gamma irradiation,[39,40,79] but these plants are not components of regular rodent chow.

Commercially available mouse enclosures specifically designed for irradiation procedures are available to protect mice undergoing irradiation from potential environmental pathogens. Using a plastic–acrylic container [for example, the RadDisk (Braintree Scientific, Braintree, MA); Figure 4 A] can be beneficial in minimizing any potential contamination between the irradiator and the irradiated mice. The container acts as a physical barrier that separates the mice from the irradiator itself (Figure 4 A, B), thus protecting the irradiator chamber from potentially infected mice. The container in Figure 3 A has a filter (pore size of 51 to 118 µm) that minimizes pathogen transmission while still providing air exchange. In contrast, the mouse restraint device shown in Figure 4 C has no filter. However, because of its relative small size, this restrainer can be used in many different irradiators, and it eliminates the issue of overcrowding because it is designed to house mice individually. Any plastic container used as a physical barrier can be sprayed with chlorinated compounds to minimize viral transmission[12] between facilities. Careful attention should be taken when spraying containers with filters, which would be rendered ineffective if they became wet. Therefore, filters should be protected during surface disinfection (for example, with a tape covering), but the protection should be removed as soon as the lid has been disinfected so as not to impede air exchange. After transplantation, recipient animals should be housed in a separate room from naïve colonies, when possible. In addition to the advantages discussed previously, this geographic separation may prevent the contamination of newly arrived naïve animals from transplanted animals that accidentally acquired pathogens from the irradiator or the laboratory.

In summary, multiple aspects of husbandry care (for example, bioexclusion practices, health monitoring, water quality, use of antibiotics) must be considered when housing mice that are undergoing irradiation or BMT. The fact that the first 7 to 10 d after transplantation are the most crucial cannot be overemphasized, and close monitoring of the recipient mice by the laboratory and husbandry staff is highly recommended to identify any possible health problems.

Murine Diseases that Can Affect BMT Studies

When immunocompromised, mice can be affected by a variety of infectious pathogens. It is crucial, therefore, that when performing BMTs, donor and recipient animals are healthy and (so far as possible) free of common viral, bacterial, and parasitic murine pathogens. The possible presence of infectious agents in the colony should be assessed at least by using sentinel animals, if not by assessment of the actual experimental animals prior to experimentation. The definition of the term 'specific pathogen-free (SPF)' varies from institution to institution based on the degree of surveillance monitoring. At our institution, mice are considered SPF if they are negative for *Ectromelia* virus, mouse rotavirus (epizootic diarrhea of infant mice), mouse hepatitis virus, mouse parvoviruses, minute mouse virus, reovirus 3, Sendai virus, Theiler murine encephalomyelitis virus, lymphocytic choriomeningitis virus, mouse adenoviruses, polyoma virus, pneumonia virus of mice, *Mycoplasma pulmonis*, cilia-associated respiratory bacillus, pinworms, and ectoparasites. Other infectious agents screened for are *Helicobacter* spp.

Maintaining mice in an SPF status for the entire course of experimentation is extremely important for reliable BMT research. Although minimizing exposure to any potential pathogen is important throughout the experimental period, the first month after BMT is particularly crucial. During this period when immune reconstitution is occuring[49,73,74] (Figure 1), mice are immunocompromised and at a greater risk of infection. In addition, careful attention must be placed to enforce strict aseptic technique when working with the cellular graft, because contamination of the bone marrow may lead to death of the recipient due to infection.[53]

The first white blood cell lineage to return to normal levels after BMT is the neutrophils. In general, human patients are not discharged from the hospital until the absolute neutrophil count is at least 1000 cells/µL (personal communication, University of Michigan bone marrow transplant unit).[37] Transplantation-related infections in humans may result from damage to the mouth, intestine, and skin from chemotherapy or radiation regimens or through indwelling catheters, augmented by the iatrogenic panleukopenia. Infections can be of bacterial, fungal, or viral origin.[18] Human herpes simplex, varicella zoster, and cytomegalovirus are some of the most common viral diseases affecting humans after BMT. Cytomegalovirus, which causes a debilitating viral pneumonia in BMT patients,[70] has been decreasing thanks to early diagnosis with PCR and the availability of new antiviral drugs. Important bacterial and fungal pathogens infecting human BMT patients include *Pseudomonas* spp., *Staphylococcus* spp., *Candida* spp., and *Aspergillus* spp.[18] Mice have a potential for contracting many of the aforementioned pathogens, especially the bacterial and fungal agents, because they are ubiquitous in the environment and are normal commensals on their skin and mucosal surfaces.[78] Human BMT patients typically are treated for at least 6 mo after engraftment with prophylactic antivirals, antibacterials, and antifungals;

in experimental murine BMT, prophylactic antimicrobials are not used frequently. Instead we rely on strict SPF status and pathogen surveillance, aseptic techniques, careful husbandry (to ensure that mice are maintained relatively 'clean'), and (in some cases) the use of antibiotic-containing or acidified water sources.

Bacterial infections affect immunosuppressed or immunocompromised mice. Some bacteria, such as *Escherichia coli*,[108] *Clostridium piliforme*,[58] and *Helicobacter* spp.,[38] have been documented to affect immunodeficient mice. Other bacterial species, such as *Staphylococcus* spp. present on the skin and mucous membranes, have been reported to affect nu/nu mice.[66,111] and naïve irradiated mice undergoing BMT,[25] causing head and neck swelling. Endogenous *Streptococcus* spp. infections can become systemic in experimentally immunosuppressed mice and have been associated with concurrent enterococcal and *Pseudomonas* spp. infections in SCID mice.[23] As a result, BMT mice may develop systemic *Pseudomonas* infections.[8,25] In other experimental BMT studies, syngeneic recipient mice infected with *Pseudomonas aeruginosa* were unable to effectively clear the *Pseudomonas* from the lungs, as a result of an impairment in macrophage phagocytosis.[74] These reports mirror the human literature, in which opportunistic bacterial species are potential pathogens for transplanted subjects.

In addition, fungal infections have been reported to affect immunocompromised mice. *Pneumocystis carinii* can exist as a saprophyte in the lungs of mice,[6] and outbreaks in SCID and nude mouse colonies have been documented.[109] Immunosuppression of previously immunocompetent carrier mice can result in *Pneumocystis* pneumonia.[9,84] Other fungal pathogens, such as *Candida albicans*, are present in the environment and can potentially affect immunodeficient mice. The potential contamination of food and bedding with fungal spores may further increase the risk of infection of immunocompromised animals. Massive fungal contaminations with *Aspergillus fumigatus, Penicillium* spp., *Fusarium* spp., and a *Cladosporium* sp. have been reported to affect experimental outcomes.[65] Dysregulation of Th1–Th2 responses occurs during BMTs,[69] and the resulting deficiency of Th1 cytokine production (which is necessary for a full immune response to *Candida albicans*) may predispose affected animals to candidiasis. Therefore, minimizing the exposure of these transiently immunosuppressed animals to fungal organisms is important and can be achieved by keeping animal cages clean, providing clean food and water, moderately lowering humidity levels (to avoid fungal growth), and using HEPA-filtered air.

As with human BMT patients, multiple reports document the effect of viruses in transplantation-related research. Murine parvoviruses, specifically murine minute virus and murine parvovirus, have been documented to suppress long-term repopulating hematopoietic stem cells,[89,91,92] cause severe leukopenia and dysregulated erythropoiesis in SCID mice,[90] potentiate allogeneic skin and tumor graft rejection,[67,68] and induce syngeneic graft rejection.[67]

In summary, bacterial, fungal, and viral diseases can adversely affect the outcomes of irradiated mice in BMT studies, causing increased morbidity and mortality in the recipient animals. Therefore minimizing the exposure of these immunosuppressed animals to potential pathogens is important. Procurement of irradiated or heat-treated food and bedding from reputable manufacturers may help to decrease the risk of contamination.[65] In addition, when animal deaths occur 7 to 21 d after transplant and are not due to expected experimental causes, viral, fungal, or other bacterial diseases may be the etiology. When faced with abnormal mortality rates, one should perform serology and pathology from moribund animals; culture the blood, tissues, and all reagents used during the experimentation of the affected transplanted animals; reassess rodent health surveillance information; reassess the details of aseptic technique; monitor animal handling procedures; and reevaluate laboratory practices. Following these guidelines will avoid or allow prompt management of potential complications after irradiation.

Institutional Care and Use Committee Considerations Regarding BMT Mice

The Institutional Animal Care and Use Committee (IACUC) has a critical role in evaluating the humaneness of animal studies involving BMT. These procedures are not innocuous; these animals typically become ill and experience considerable weight loss after irradiation and transplantation. The application for animal use should convey to the IACUC the expected outcomes of the various procedures in the research, the necessary endpoints of the studies, and any considerations for possible exemption from institutional standards for euthanasia. Investigators also need to describe their monitoring procedures adequately so that the IACUC can be assured that animal distress is minimized, and when present, is only associated with the scientific goals of the project. The IACUC should require these descriptions if not provided in the initial application.

Monitoring the body weight of laboratory animals is a typical method of limiting the severity of the experimental procedures. Some institutions have established maxima for tolerable weight loss; we are aware of 15% and 20% limits being used at local institutions as a rationale for euthanasia before the intended experimental endpoint. Animals that undergo irradiation for BMT typically lose a considerable amount of weight, only to gain it back relatively quickly after successful transplantation. At our institution, weight loss of 20%, or greater is not uncommon after irradiation (Figure 6). The extent of the loss depends on the animals' age, weight, strain or genetic line, and experimental factors. Therefore, investigators using BMT procedures may request that their animals be permitted a weight loss greater than 20%, despite local animal welfare policies.

Any request to permit significant weight loss without euthanasia must include a plan for monitoring the overall health of the animals. In general, more than 90% of syngeneic transplanted animals should recover their weight loss within 14 d after irradiation. Therefore, BMT mice should be monitored at least once, if not twice, daily for the first 14 d, with body weights obtained at least every other day. If recipient mice still look severely ill, or are still losing body weight by 10 to 14 d after BMT, they should be examined by the veterinary staff or euthanized by the laboratory staff. Monitoring plans such as these are necessary components of an IACUC application that requests exceptions to standard weight loss guidelines.

An alternate measure that can be used to evaluate mice after experimental irradiation and transplantation is body condition scoring,[101] which is essentially a subjective comparison of muscle mass and body fat to the skeletal structure and is used to evaluate the general health of animals when body weight alone is a poor indicator. For example, body condition scoring often is used for tumor-bearing or ascites-producing mice, because these animals can gain total body weight (due to the accumulation of neoplastic tissue and fluid, respectively) while actually losing muscle mass. Although body weight is a good indicator of general health for BMT mice, monitoring the body condition score can aid in determining the overall health status of the recipient mice. In addition, overweight mice tend not to recover

their original body weight after transplant (Figure 6), but they do appear healthy otherwise after recovery, and this situation can be assessed through body condition scoring. Therefore, requiring assessment and recording of body condition scores, in addition to body weight, is a reasonable requirement that could be imposed by the IACUC.

Summary

Animal models have been important for the discovery and development of many of the BMT techniques and therapies currently used in hospitals. These advances have benefitted both human and animal health, because BMT is starting to be used in companion animals.[62] During the discovery period, when mouse experimentation is necessary, an understanding of the sequence and intended outcomes of the procedures performed in the transplanted mice is necessary, as is knowledge of the means available to minimize unwanted side effects. Maintaining open communication between the laboratory staff, veterinary teams, and the IACUC is extremely important to enable high-quality research, ensure the minimization of animal distress and unnecessary peritransplant complications, and guarantee the ethical use of animals in compliance with reduction, refinement, and replacement principles.

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